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(54) Title: PURIFICATION OF OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES USING ANION EXCHANGE CHROMATOGRAPHY

(57) Abstract

The present invention provides improved methods for separating and purifying oligonucleotide phosphorothioates. For the first time, we used DMAE ion-exchange chromatography to separate and purify oligonucleotide phosphorothioates. We found that by varying the conditions employed, excellent separation can be obtained. In particular, we have been able to use a higher pH and lower salt concentrations than previously reported in the literature to obtain chromatographic separation of oligonucleotide phosphorothioates having length up to about 35 nucleotides.

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PURIFICATION OF OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES USING ANION EXCHANGE CHROMATOGRAPHY

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the field of purification of oligodeoxynucleotide phosphorothioates.

Description of the Prior Art

The use of modified phosphate backbone oligodeoxynucleotides as antisense oligonucleotides in the field of selective gene regulation for therapeutic purposes has received increasing attention over the last several years. There are numerous types of modified phosphate linkages, e.g., methylphosphonate, phosphorothioate, phosphoramidate, that have been incorporated into antisense oligonucleotides and studied. *E.g.*, Erickson and Izant (Eds.), *Gene Regulation: Biology of Antisense RNA and DNA* (Raven Press, New York, 1992). Oligodeoxyribonucleotide phosphorothioates, for example, have been found to inhibit immunodeficiency virus (Agrawal et al., *Proc. Natl. Acad. Sci. Usa* **85**, 7079 (1988); Agrawal et al.,*Proc. Natl. Acad. Sci. USA* **86**, 7790 (1989); Agrawal et al., in *Advanced Drug Delivery Reviews* **6**, 251 (R. Juliano, Ed., Elsevier, Amsterdam, 1991); Agrawal et al. in *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS*, 143 (E. Wickstrom, Ed., Wiley/Liss, New York, 1991); Zamecnik and Agrawal in *Annual Review of AIDS Research*, 301 (Koff et al., Eds., Dekker, New York, 1991); and Matsukura et al., *Proc. Natl. Acad. Sci. USA* **83**, 4244 (1988)), and influenza virus (Leiter et al., *Proc. Natl. Acad. Sci. USA* **87**, 3420-3434 (1990)) in tissue culture. In addition, oligodeoxyribonucleotide phosphorothioates have been the focus in a wide variety of basic research (*e.g.*, Agrawal et al., *Proc. Natl. Acad. Sci. USA* **87**, 1401 (1990) and Eckstein and Gish, *Trends Biochem. Sci.* **14**, 97 (1989)), enzyme inhibition studies (Mujumdar et al., *Biochemistry* **28**, 1340 (1989)), regulation of oncogene expression (Reed et al., *Cancer Res.* **50**, 6565 (1990)) and IL-1 expression (Manson et al., *Lymphokine Res.* **9**, 35 (1990)) in tissue culture.

Automated synthesizers have proven an invaluable tool for obtaining oligonucleotides. Oligonucleotides are produced stepwise, with the addition of one monomer at a time to the nascent oligonucleotide chain. 2-3% of the reactions fail, however, during each cycle in which a nucleotide monomer is to be added. Consequently, the resulting products are generally a heterogenous mixture of oligonucleotides of varying length. For example, in a typical 20mer synthesis, the 20mer product represents only 50-60% of the recovered oligonucleotide product. For most purposes (e.g., therapeutic) the purity of the compounds is extremely important. Consequently, there has been an interest in developing chromatographic techniques for purifying oligonucleotides. Because of their therapeutic potential, much of the focus has been on purifying oligonucleotide phosphorothioates.

Methods of oligodeoxynucleotide phosphorothioate purification have been published. Agrawal et al., *J. Chromatography* **509**, 396 (1990), reported the analysis of oligonucleotide phosphorothioates using high-performance liquid chromatography with a reverse-phase column. In that study, Agrawal et al. converted the oligonucleotide phosphorothioate to its phosphodiester counterpart and then carried out HPLC analysis. Using this method they were able to analyze oligonucleotide phosphorothioates containing 10 or fewer nucleotides on a strong anion-exchange column (Partisphere SAX column). Oligonucleotide phosphorothioates having more than 10 nucleotides could not be analyzed, however, because of the strong interaction with the SAX medium.

Metelev and Agrawal, *Anal. Biochem.* **200**, 342 (1992), reported the ion-exchange HPLC analysis of oligodeoxy-ribonucleotide phosphorothioates on a weak anion-exchange column (Partisphere WAX) in which the weak anion exchanger utilizes a dimethylaminopropyl functional group bonded to Partisphere silica. This medium, with an ion-exchange capacity of 0.18 meq/g, exhibits an interaction with anions weaker than those observed with strong anion-exchange media. The authors of this study found that separation was length dependent for oligonucleotide phosphorothioates up to 25 nucleotides in length. Furthermore, n-1 peaks were well separated from the parent peak. They also found that 30-mer and 35-mer oligonucleotide phosphorothioates were separable with the same gradient, although better separation could be obtained with a shallower gradient.

Metelev et al., *Ann. N.Y. Acad. Sci.* **660**, 321-323 (1992), reported the analysis of oligoribonucleotides and chimeric oligoribo-oligodeoxyribonucleotieds using ion-exchange HPLC. They found that the retention time of the oligonucleotides studied depended on the number of ribonucleotide moieties in the oligonucleotide. In addition, the retention time of oligoribonucleotides was found to be length dependent. The authors noted that oligoribonucleotides of length up to 25 nucleotides could be purified and analyzed.

Bigelow et al., *J. Chromatography* **533**, 131 (1900), reported the use of ion-pair HPLC to analyze oligonucleotide phosphorothioates. Stec. et al., *J. Chromatography* **326**, 263 (1985), and Agrawal and Zamecnik, *Nucleic Acids Res.* **19**, 5419 (1990), reported HPLC analysis of oligodeoxyribonucleotides containing one or two phosphorothioate internucleotide linkages using a reversed-phase column.

These methods of oligonucleotide phosphorothioate purification use HPLC. While this technique is useful for small scale operations, it is unsuitable for large, commercial scale use.

SUMMARY OF THE INVENTION

An object of the present invention is to provide improved methods for purifying oligodeoxynucleotide phosphorothioates. In particular, an object of the invention is to provide purification techniques suitable for large scale separation of oligonucleotide phosphorothioates. We have achieved these objectives by developing methods based on several discoveries. First, we have developed a method of chromatography using a DMAE Fractogel EMD column whereby we have eliminated the necessity of using an elution buffer containing organic solvents. This method is also advantageous because it does not require elevated temperatures, making it more amenable for large scale chromatography.

Another improvement that we have developed is that the methods of the present invention use an elution buffer having a salt concentration that is significantly lower than has been previously known in the art. The principal advantage this discovery confers is to allow for separation of longer oligodeoxynucleotide phosphorothioates in ion exchange columns.

The methods disclosed herein provide the further advantage of being useful for large scale purification of oligodeoxynucleotide phosphorothioates. These methods can replace the conventional C-18 protocol.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effects of different solvent system on the elution of GEM 91.

Figure 2 shows the separation of 15mer, 20mer and 25mer on an ion exchange column (medium particle size 25-40 μm).

Figure 3 shows separation of 24mer and 25mer oligonucleotides on a DMAE Fractogel EMD column.

Figure 4 shows purification of GEM-91 (SEQ ID NO 1) on a DMAE Fractogel EMD ion exchange column.

Figure 5 shows polyacrylamide gel electrophoretic analysis of fractions shown in Figure 4.

Figure 6 shows capillary gel electrophoretic analysis of fraction D shown in Figure 5.

Figure 7 shows the purification of GEM-91 (GEM-91 (SEQ ID NO 1)) on an ion exchange column using 2.5 cm internal diameter column and the same buffer system as described in Figure 2.

Figure 8 shows polyacrylamide gel electrophoretic analysis of fractions shown in Figure 7; electrophoresis conditions were the same as described in Figure 5.

Figure 9 shows purification of GEM on an ion exchange column with an inner diameter of 5 cm).

Figure 10 shows purification of GEM 91 on an ion exchange column using high pH buffer system.

Figure 11 shows purification of HOFF (SEQ ID NO 3) on DMAE Fractogel EMD ion exchange column using high pH buffer system; described above in Figure 10.

Figure 12 shows comparison of the purification of GEM-91 (GEM-91 (SEQ ID NO 1)) on different particle sizes; (A) particle size was 25-40 μm, (B) 45-90 μm.

Figure 13 shows purification of GEM-91 (GEM-91 (SEQ ID NO 1)) on 2.6 cm ID of DMAE Fractogel EMD (particle size 45-90) column using high pH buffer system.

Figure 14 shows polyacrylamide gel electrophoretic analysis of the fractions shown in Figure 13.

Figure 15A shows capillary gel electrophoretic analysis and Figure 15B shows GEN-FAX ion exchange chromatographic analysis of the pooled fractions (fraction #3-9) shown in Figure 13.

Figure 16 shows purification of HOFF (SEQ ID NO 3) sample on a 2.6 cm ID DMAE Fractogel ion exchange column; the buffer conditions used here were the same as described in Figure 13.

Figure 17 shows electrophoretic analysis of fractions shown in Figure 16.

DETAILED DESCRIPTION OF THE INVENTION

The present invention comprises methods for separating or purifying oligonucleotide phosphorothioates by ion-exchange chromatography. As used herein, the terms "separating" and "purifying" are intended to be used interchangeably and mean a process by which oligonucleotides having a particular molecular structure are physically segregated from oligonucleotides have a different molecular structure. In particular, the present invention comprises the use of DMAE ion exchange chromatography to separate oligonucleotide phosphorothioates.

The interaction between oligodeoxynucleotide phosphorothioates and the column medium is complicated. Phosphorothioates not only interact with the ion exchanger resin, but with the matrix also. The phosphorothioates bind to the medium more tightly than do phosphodiesters, and thus need higher salt concentrations to elute.

Chromatography performed in elevated temperatures results in prolonged retention time of oligonucleotide phosphorothioates, indicating that the conformation of the compounds changes under different chromatography conditions. Oligodeoxynucleotide phosphorothioates become stretched at elevated temperatures, thereby exposing more negative charges on the surface of the molecules.

Considering the complexity of the interaction between the phosphorothioates and the column medium, it was thought that a high pH elution buffer could improve the purification of the phosphorothioates by loosening the binding of phosphorothioates to the DMAE exchanger. Hence, the experiments disclosed herein were designed to use stronger, high pH buffer. In addition, a higher linear flow rate was employed to prevent the size exclusion effect of the tentacle moiety. To simplify the process, no organic solvents or additives were used in the elution buffer

We have found that oligonucleotide phosphorothioates can be purified on ion-exchange columns using aqueous elution buffers having a higher pH and lower salt concentration than has previously been reported. The results presented below demonstrate that the purification techniques described can resolve oligonucleotides differing in length by one nucleotide. The protocols presented herein can be employed in large scale purification of oligonucleotide phosphorothioates to replace

the conventional C_{18} protocol. This results in fewer steps in the manufacturing process and allows for better purity and recovery of product.

The DMAE Fractogel EMD medium has a pK value about 9. The manufacturer of the medium suggests that the pH value of the elution buffer used should not exceed 8. Contrary to this warning, we have found that separations of oligonucleotide phosphorothioates having length of about 10 nucleotides to about 35 nucleotides are best performed with an elution buffer having a pH of greater than 8.

According to the method of the present invention, oligonucleotide phosphorothioates from about 10 to about 35 nucleotides in length can be separated on a DMAE ion exchange column. A polyethyleneimide column may also be used. In a preferred embodiment, oligonucleotide phosphorothioates having a length of from about 25 to about 35 can be separated using the present methods. The oligonucleotides separable by the present method may have as few as one and as many as all phosphorothioate internucleotide linkages. Oligonucleotide phosphorodithioates of the same size as the oligonucleotide phosphorothioates described can also be separated by the same methods.

The oligonucleotides are placed on the column and eluted at or near ambient (room) temperature with a gradient of a first buffer (buffer A) comprising about 25 mM Tris-HCl and about 20% 50 mM aqueous mannitol and a second buffer (buffer B) comprising buffer A and about 2 M NaCl (or another suitable salt). It is found that oligonucleotide phosphorothioates will elute at salt concentrations of less than about 2 M and often less than about 0.5 M.

The separation method of the present invention is essentially independent of column particle size. Sizes ranging from 25 to 90 μm can be used successfully. In two preferred embodiment, the particle size is 25 μm - 40 μm or 45 μm - 90 μm.

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention.

EXAMPLES

All oligodeoxynucleotide phosphorothioates purified according to the method of the invention were synthesized on an automated synthesizer (MilliGEN 8700 or 8800 DNA synthesizer) using standard conditions. Their sequences are listed in TABLE 1 below.

TABLE 1

NAME	SEQ ID NO:	SEQUENCE	TYPE
GEM-91	1	CTCTCGCACCCATCTCTCTCCTTCT	25mer
GEM-92	2	CTCTCGCACCCATCTCTCTCCTTCTGGAGAGAG	32mer
HOFF	3	GAGGGGAAACAGATCGTCCATGGT	23mer
PAD-24	4	ACACCCAATTCTGAAAATGGGCAT	24mer
PAD-15	5	CTCTCGCACCCATCT	15mer
PAD-20	6	CTCTCGCACCCATCTCTCTC	20mer

We employed DMAE Fractogel EMD, manufactured by E. Merck Separation (Earmstat, Germany) in these experiments. The Fractogel matrix, copolymerized from oligoethyleneglycol, glycidlmethacrylate, and pentaerythroldimethacrylate combines a hydrophilic surface with mechanical stability and chemical resistance at extreme pH values. The tentacle ion exchange moieties are exclusively on linear polyelectrolyte chains grafted on the support.

Since an aqueous system is more suitable than an organic aqueous solvent in pharmaceutical manufacturing processes and less salt is needed to elute the oligodeoxynucleotide phosphorothioates, a Tris-HCl plus mannitol buffer was chosen here for the purification of GEM-91 (SEQ ID NO 1). Mannitol may act as a displacement factor.

Example 1

Effect of Different Solvent Systems on Optimum Salt Concentrations

GEM-91 (SEQ ID NO 1) (trityl-off), purified on C_{18} reverse phase chromatography with trityl-on and then detritylated, was loaded on a DMAE (dimethylaminoethyl) Fractogel EMD (particle size 25-40 μm) ion exchange column having inner diameter of 1.5 cm and a flow rate of 5 ml/min. The following buffer systems were used to test the effect of different solvent systems on the optimum salt concentration:

1) Buffer A — 25 mM Tris-HCl (pH 8.0) containing 20% acetonitrile;
Buffer B — Buffer A + 2M NaCl.

2) Buffer A — 25 mM Tris-HCl (pH 8.0) containing 20% 50 mM aqueous solution of mannitol;
Buffer B — Buffer A + 2 M NaCl.

Table 2 presents the elution gradient used.

TABLE 2

Time (minutes)	Gradient
0 - 2	isocratic 20% Buffer B
2 - 50	20% Buffer B to 80% Buffer B

Figure 1A shows the elution profile when buffer system 1 was used, and Figure 1B shows the elution profile when buffer system 2 was used. The results show that GEM-91 (SEQ ID NO 1) eluted at a salt concentration of about 1.1 M NaCl in buffer system 1, while in buffer system 2 it eluted at a lower salt concentration, about 1 M NaCl.

We chose the Tris-HCl/mannitol solution because an aqueous system is more suitable than organic-aqueous solvent in pharmaceutical manufacturing. The effect of mannitol on the elution of GEM-91 (SEQ ID NO 1) is unknown, except that mannitol may act as a displacement factor.

Example 2

Length-Based Resolution of Oligonucleotide Phosphorothioates

A mixture of GEM-91 (SEQ ID NO 1) (15mer), PAD-15 (SEQ ID NO 5) (20mer), and PAD-20 (SEQ ID NO 6) (25mer) was loaded on a 1.5 cm ID DMAE Fractogel EMD exchange column (particle size 25-40 μm) and eluted with a linear flow rate of 5 ml/min. The following buffer system was used:

Buffer A — 25 mM Tris-HCL (pH 8.0) containing 20% 50 mM aqueous solution of mannitol;

Buffer B — Buffer A + 2 M NaCl.

Table 3 presents the gradient used.

TABLE 3

Time (minutes)	Gradient
0 - 5	isocratic 5% Buffer B
5 - 35	linear gradient from 15% to 32% buffer B
35 - 40	isocratic 32% buffer B
40 - 70	linear gradient from 32% to 50% buffer B

Figure 2 presents the results. The retention times were 29.66 minutes for the 15mer, 42.14 minutes for the 20mer, and 52.67 minutes for the 25mer. These results show that this method is capable of fully resolving (separating) oligonucleotide phosphorothioates differing in length by five nucleotides when the overall oligonucleotide length is between 15 and 25 nucleotides.

The same conditions were used with a mixture of GEM-91 (SEQ ID NO 1) (25 mer) and PAD-24 (SEQ ID NO 4) (24mer). Figure 3 presents the results, showing that this method is capable of partially separating oligonucleotide phosphorothioates differing in length by only one nucleotide.

Example 3

Purification of GEM-91 (SEQ ID NO 1)

We purified a crude sample of GEM 91 (SEQ ID NO 1) (60 A_{260} units), which contained 50% produt using the same methods described in Example 2. Fractions containing GEM-91 (SEQ ID NO 1) were collected (Figure 4) and analyzed with PAGE gel (20% of gel, run under constant current of 15 mA, Figure 5) and capillary gel (Figure 6) electrophoresis. The purity of the final product was 88% based on CE analysis. The amount of final product was about 30% of the crude sample.

Example 4

Large Scale Chromatography

Scale-up experiments were carried out to determine whether ion exchange chromatography is suitable for manufacture processes. GEM-91 (SEQ ID NO 1) was separated from a crude mixture with a 2.5 cm diameter column and the same buffer systems as in Example 2 with a flow rate of 10 ml/min. Table 4 presents the gradient used.

TABLE 4

Time (minutes)	Gradient
0 - 5	isocratic 15% Buffer B
5 - 40	linear gradient from 15% to 32% buffer B
40 - 45	isocratic 32% buffer B
45 - 80	linear gradient from 32% to 45% buffer B

Figure 7 presents the results. The final product had a purity of about 85%; the recovery was about 30%. PAGE analysis showed that the rear fractions contained more n-1 failure sequence than front fractions (Figure 8), possibly resulting from the linear flow rate. The linear flow rate employed in the column (2.04 cm/min) was lower than in the small column (1.5 cm diameter), which had a flow rate 10 ml/min. Under low linear flow rate conditions, the tentacle moiety of the column medium causes the

column to behave as a size exclusion chromatography column, causing late elution of n-1 failure sequence.

A large amount of crude GEM-91 (SEQ ID NO 1) sample (100,000 A_{260} units) was purified on a 5 cm diameter column using the same buffer system as in Example 2, with a flow rate of 20 ml/min. Table 5 presents the gradient used.

TABLE 5

Time (minutes)	Gradient
0 - 5	isocratic 15% Buffer B
5 - 60	linear gradient from 15% to 32% buffer B
60 - 120	isocratic 32% buffer B
120 - 160	linear gradient from 32% to 45% buffer B
160 - 165	isocratic 45% buffer B
165 - 260	linear gradient from 45% to 100% buffer B

Figure 9 presents the results. The purity of the final product was about the same as on 2.5 cm diameter column, but the recovery was only 25%. The rear fractions also contained more n-1 failure sequence. More salts were needed to elute GEM-91 (SEQ ID NO 1) (about 1.3 M NaCL instead of 0. 8 M) . The linear flow rate employed in this column was lowered to 1.02 cm/min due to the limitation of back pressure. The insufficient volume of eluent caused by low linear flow rate required high concentration of NaCl to elute GEM-91 (SEQ ID NO 1).

HOFF (SEQ ID NO 3) (24mer) samples rich in guanidine could not be eluted with Tris-HCl, mannitol at 2 M NaCl, even when the solution contained saturated urea.

Example 5

Effect of Elution Buffer pH

To test the theory that an elution buffer having a higher pH than that recommended by the manufacturer of the DMAE Fractogel EMD column could be used,

we placed a crude sample of GEM-91 (SEQ ID NO 1) on a 1.0 cm diameter column (25 - 40 μm particle size) with a flow rate of 5 ml/min (corresponding to a liner flow rate of 6.39 cm/min). The following buffer system was used:

Buffer A: 50 mM Tris-HCl, pH 9

Buffer B: buffer A + 2 M NaCl.

Table 6 presents the gradient used.

TABLE 6

Time (minutes)	Gradient
0 - 2	isocratic 0% Buffer B
2 - 40	linear gradient from 0% to 15% buffer B

The compound eluted at about 200 mM NaCl (Figure 10). Although the peak of GEM-91 (SEQ ID NO 1) was fairly broad, the amount of the product was close to that calculated from the synthesis yield.

Crude HOFF (SEQ ID NO 3) (24mer) sample can also be separated under the same conditions. The retention time of the final product is almost the same as that of GEM-91 (SEQ ID NO 1) (Figure 11).

A larger column (2.6 cm ID, particle size 45-90 μm) was usd for a scale-up experiment. The buffer system used was as follows:

Buffer A: 50 mM Tris-HCl, pH 9.0

Buffer B: buffer A + 1 M NaCl.

Table 7 presents gradient used with a flow rate of 35 ml/min:

TABLE 7

Time (minutes)	Gradient
0 - 5	isocratic 0% Buffer B
5 - 60	Linear gradient from 0 to 30% Buffer B
60 - 61	Linear gradient from 60% to 100% buffer B

The main peak of the GEM-91 (SEQ ID NO 1) sample was fractionated into 9 different fractions (Figure 13). Each fraction and the latest peak were analyzed by PAGE. Less failure sequences were observed in fraction #4-#9. No n-1 failure sequences were present in later fractions (Figure 14). This suggests that the higher linear flow rate (6.6 cm/min) may prevent the size exclusion effect of the tentacle moiety. The data also show that the latest peak contains mostly n+x sequence, which may be caused in the synthesis and can not be separated using a C18 reverse phase column (Figure 14).

Fractions #3-#9 were pooled and analyzed on a GENFAX anion exchange column (Figure 15A) and by capillary electrophoresis (Figure 15B). The purity of the final product was higher than 90% and the recovery was 50% of the starting material. SEQ ID 3 (24mer) can be purified from the crude sample using the same column gradient (Figure 16). However, the three main peaks contained main products (Figures 16 and 17), indicating that base stacking may occur during separation.

Example 6

Effect of Column Medium Particle Size

In this experiment a 1.0 cm diameter column was used with the following buffer system:

Buffer A: 50 mM Tris-HCl, pH 9.0

Buffer B: buffer A + 1 M NaCl, with a flow rate of 5 ml/min.

Table 8 presents the gradient used.

TABLE 8

Time (minutes)	Gradient
0 - 5	isocratic 0% Buffer B
2 - 50	linear gradient from 0% to 20% buffer B

The results of using this system with two different column medium particle sizes are displayed in Figures 12A (25 µm - 40 µm particle size) and 12B (45 µm - 90 µm). As can be surmised from a comparison of the Figures, the change in particle size had no significant effect.

SEQUENCE LISTING

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..25
(D) OTHER INFORMATION: /note= "All internucleotide linkages are phosphorothioates"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCTCGCACC CATCTCTCTC CTTCT 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..33
(D) OTHER INFORMATION: /note= "All internucleotide linkages are phosphorothioates"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTCTCGCACC CATCTCTCTC CTTCTGGAGA GAG 33

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /note= "All internucleotide linkages are phosphorothioates"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAGGGGAAAC AGATCGTCCA TGGT 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

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(A) NAME/KEY: -
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACACCCAATT CTGAAAATGG GCAT 24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /note= "All internucleotide linkages are phosphorothioates"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCTCGCACC CATCT 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /note= "All internucleotide linkages are phosphorothioates"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTCTCGCACC CATCTCTCTC 20

What is claimed is:

1. A method of purifying oligonucleotide phosphorothioates by DMAE anion-exchange chromatography comprising eluting the oligonucleotide with an aqueous elution buffer gradient including mannitol and having a pH greater than about 8.0, wherein the oligonucleotide has a length of about 10 to about 35 nucleotides, at least one phosphorothioate internucleotide linkage, and elutes at a salt concentration of less than about 2 M.

2. A method of purifying oligonucleotide phosphorothioates by anion-exchange chromatography according to claim 1 wherein the oligonucleotide phosphorothioates elute at a salt concentration of less than about 0.5 M.

3. A method of purifying oligonucleotide phosphorothioates by anion-exchange chromatography according to claim 1 wherein the oligonucleotide phosphorothioates have length of about 25 to about 35 nucleotides.

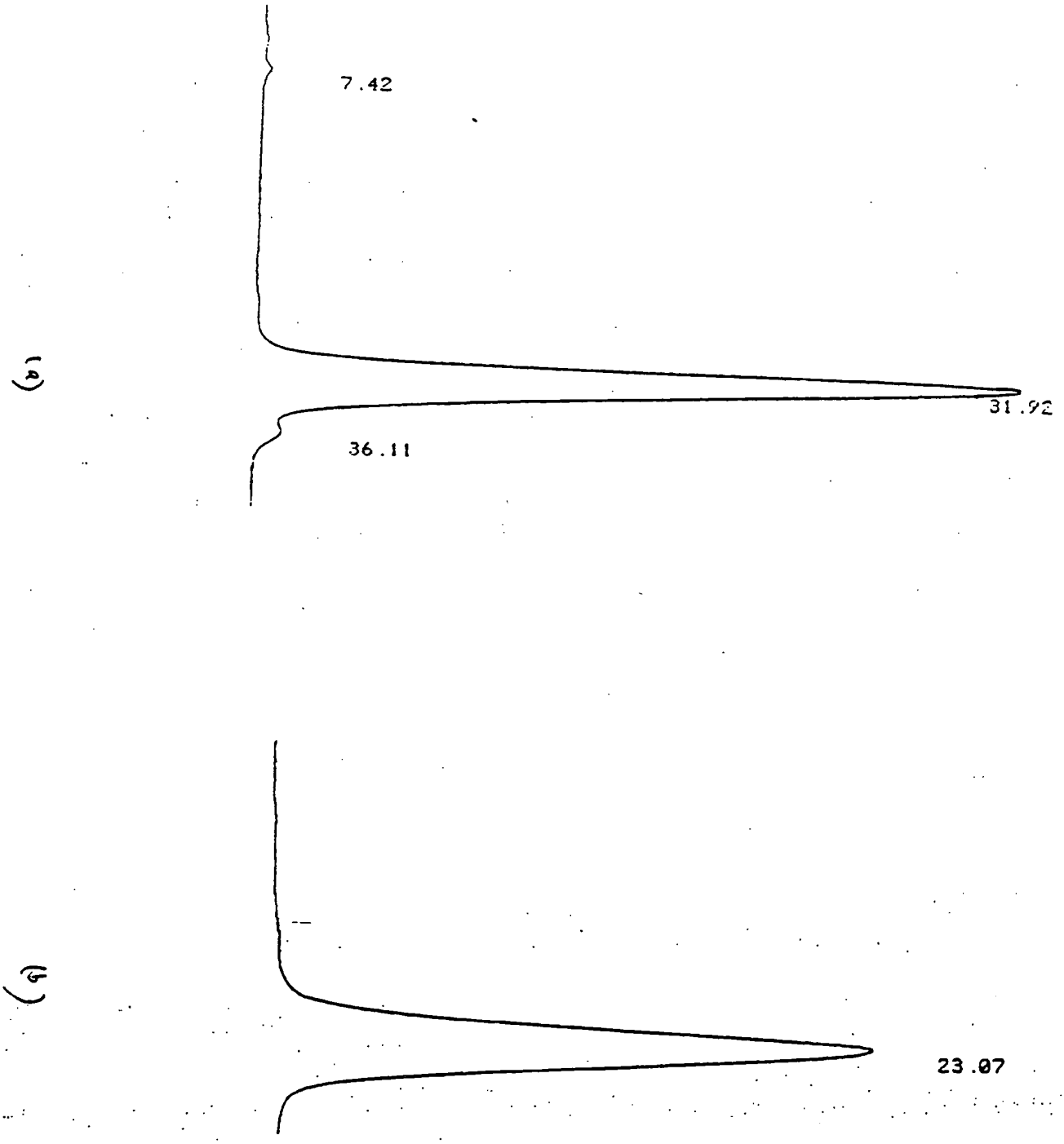


FIG. 1

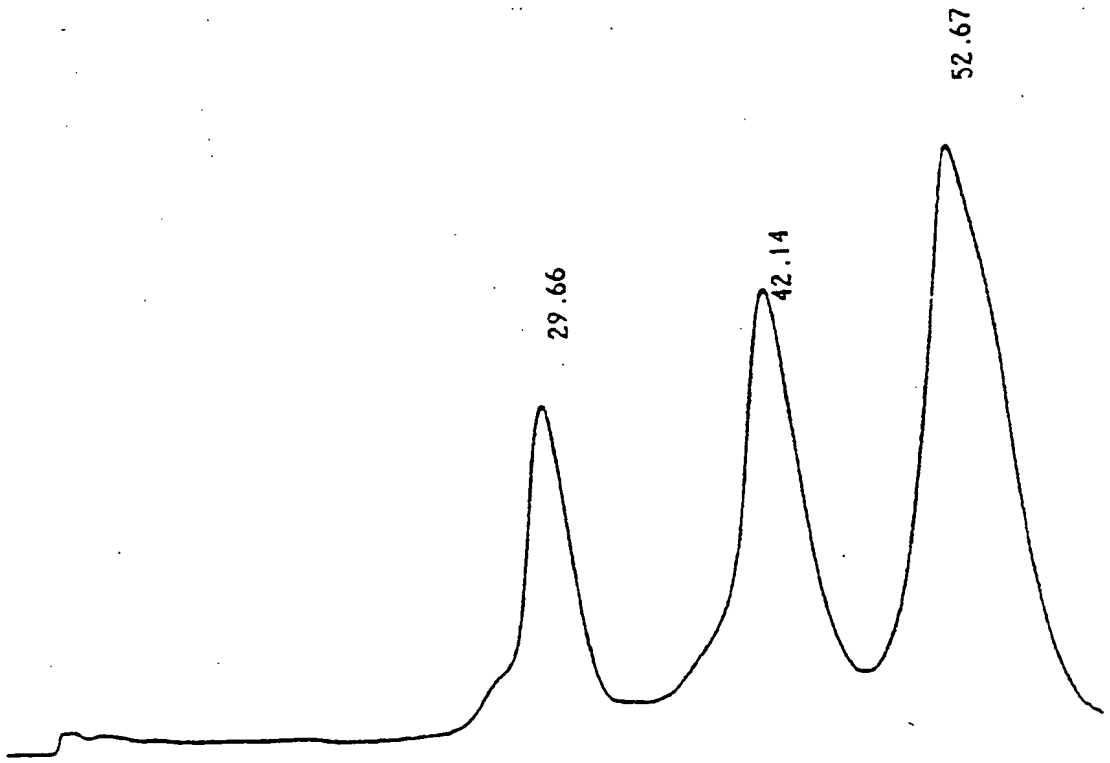


FIG. 2

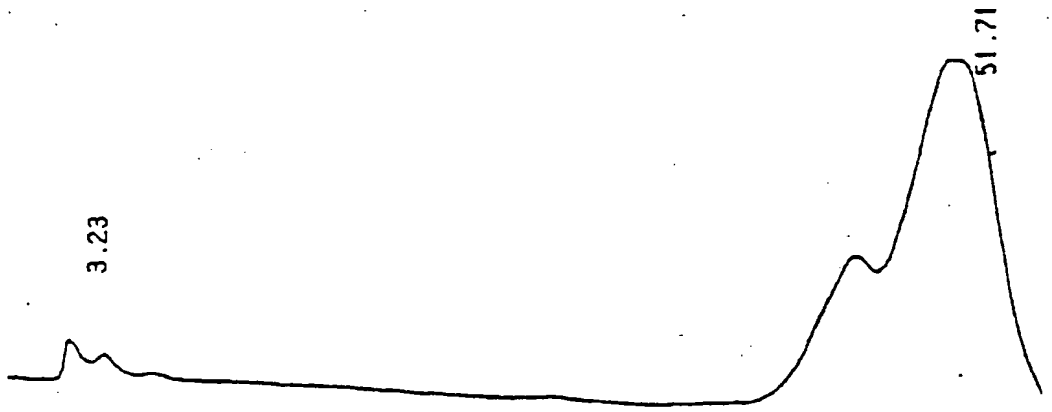


FIG. 3

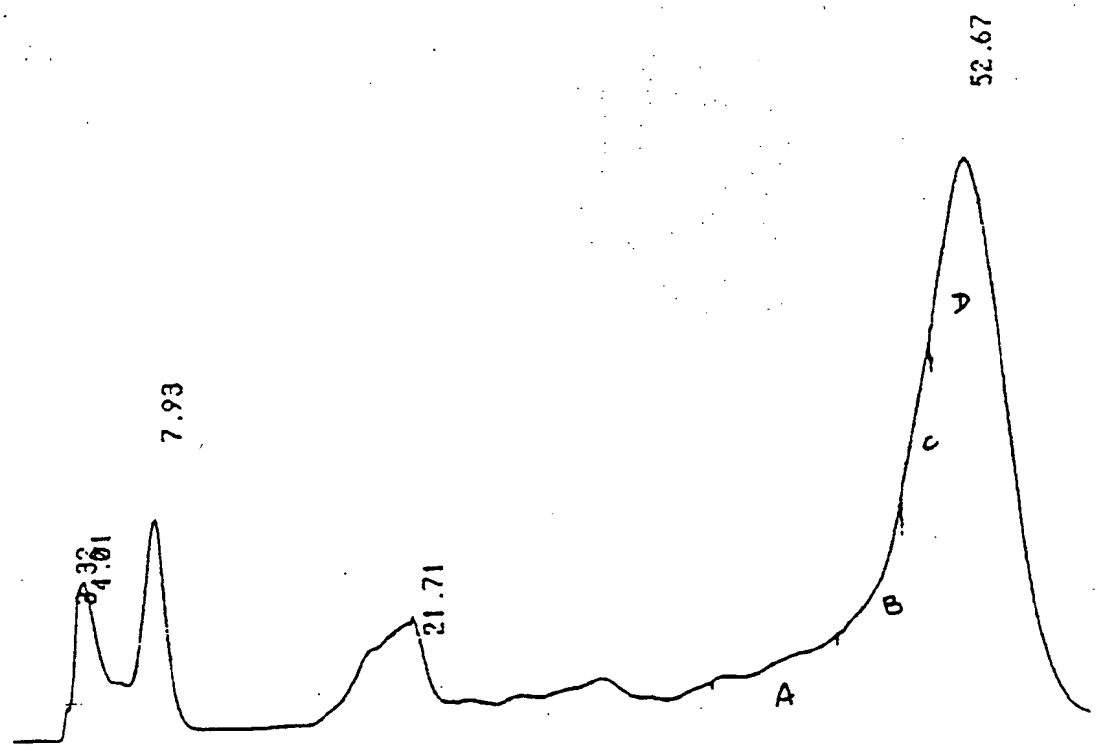


FIG. 4

FIG. 5

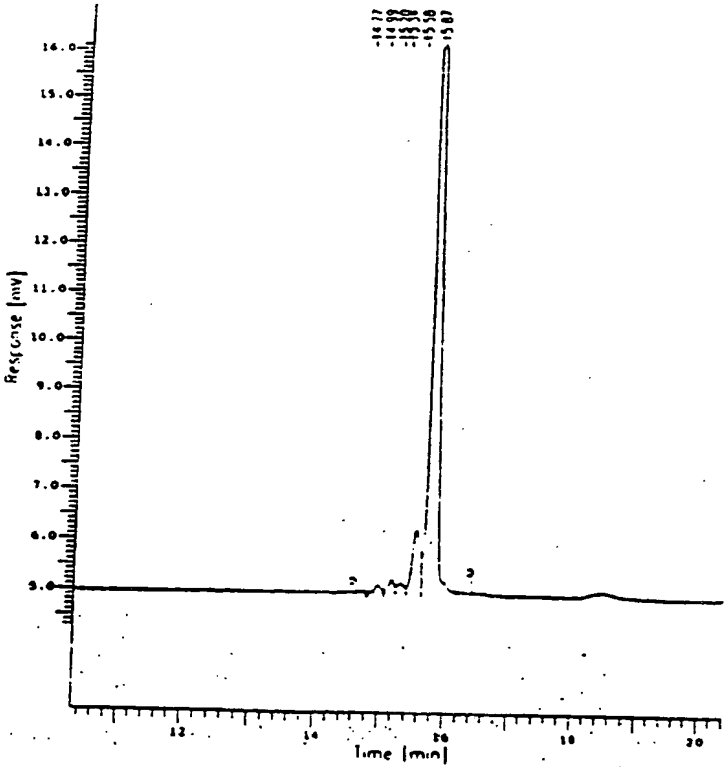


FIG. 6

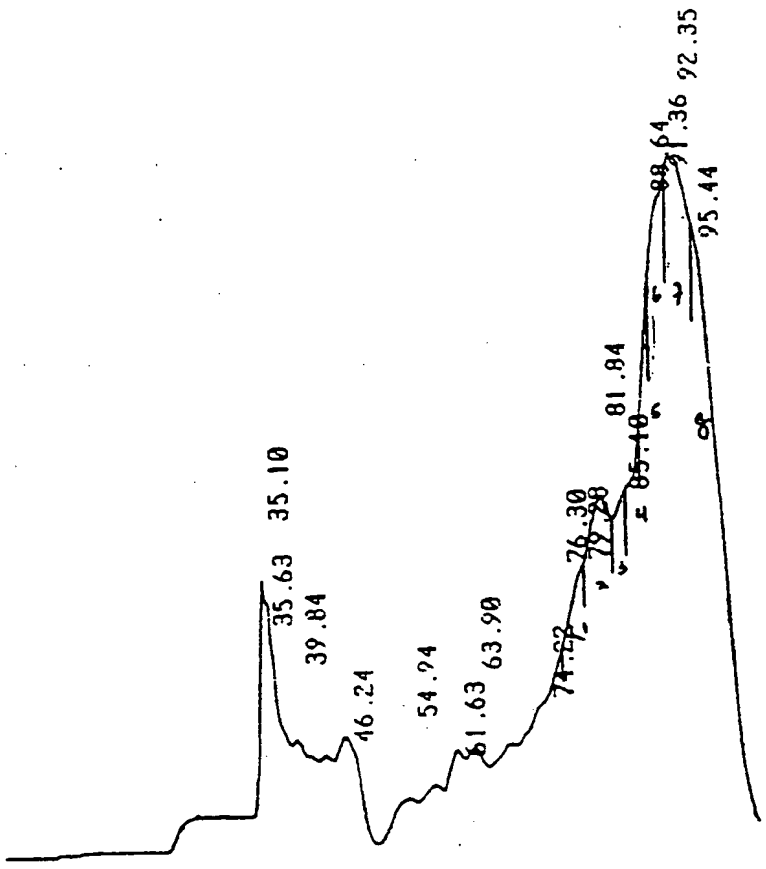

35.10
35.63
39.84
46.24
54.24
61.63
63.90
74.62
76.30
81.84
85.10
92.35
95.44

FIG. 7

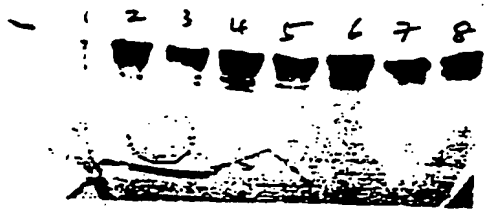


FIG. 8

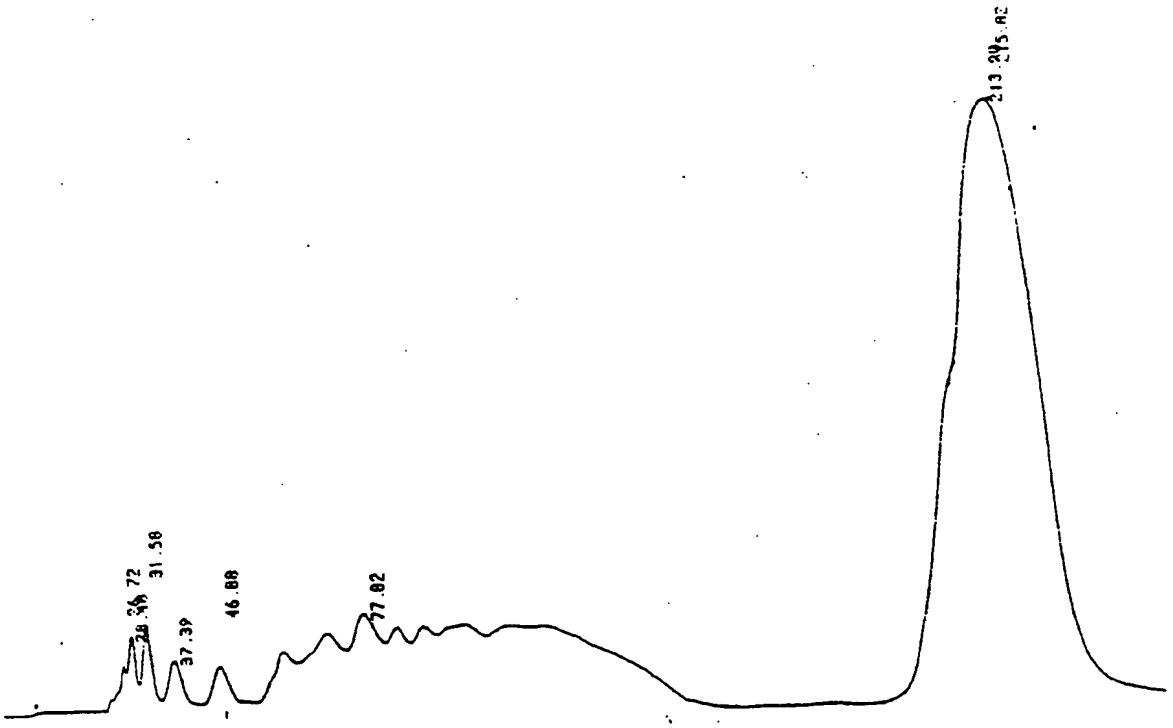


FIG. 9

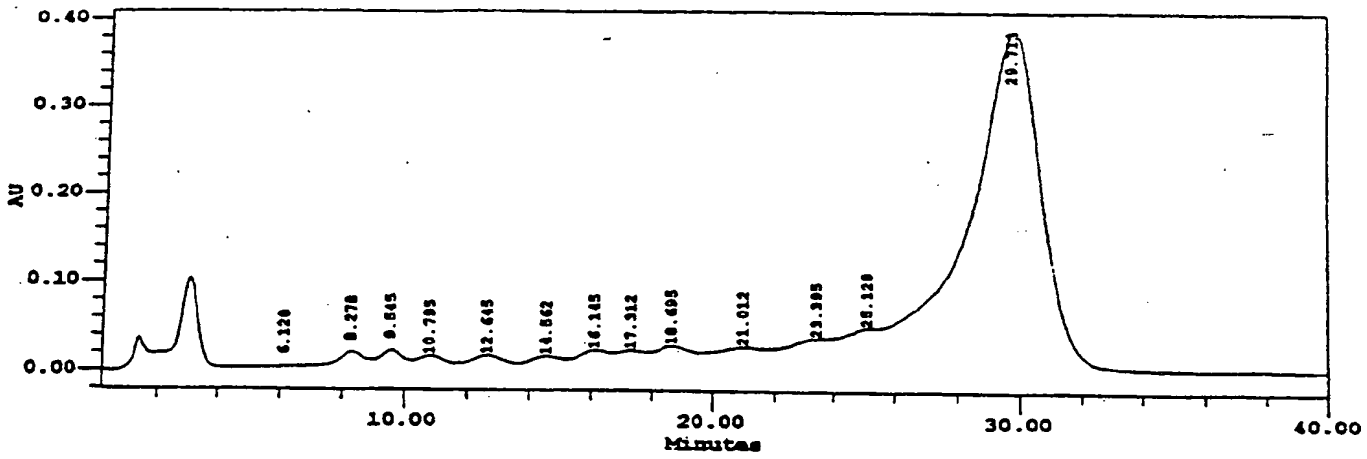

0.40
0.30
0.20
0.10
0.00
AU
10.00
20.00
30.00
40.00
Minutes

FIG. 10

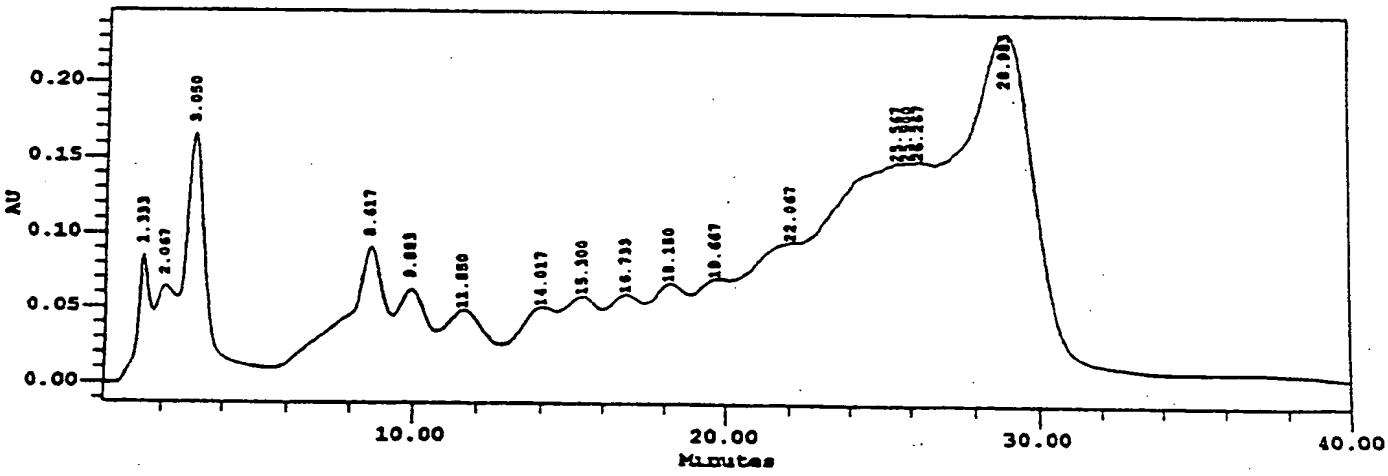
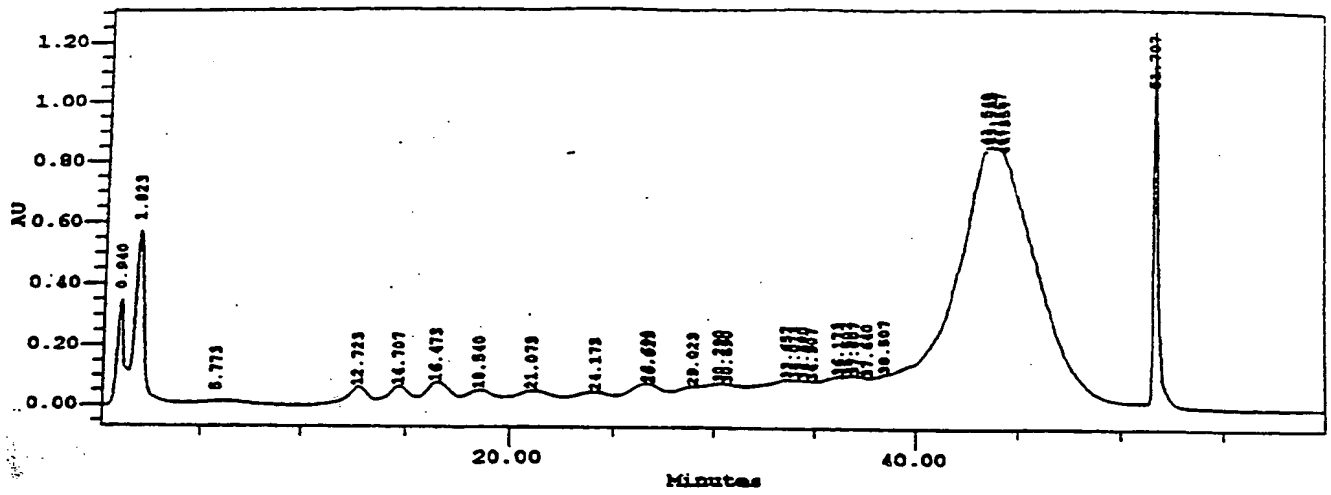

0.20
0.15
0.10
0.05
0.00
AU
10.00
20.00
30.00
40.00
Minutes

FIG. 11


a)
AU
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.840
1.023
8.773
12.723
14.707
16.473
18.840
21.073
24.173
26.688
29.023
41.307
20.00
40.00
Minutes

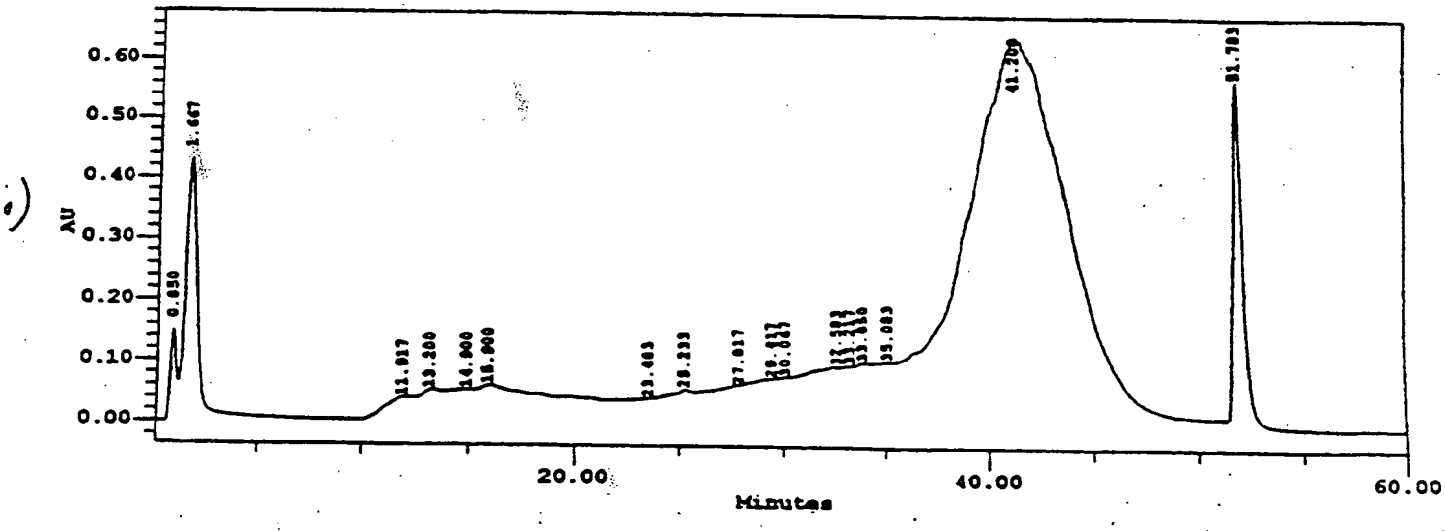

b)
AU
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.830
1.667
11.817
13.200
14.900
16.900
23.483
28.233
37.017
41.700
51.783
20.00
40.00
60.00
Minutes

FIG. 12

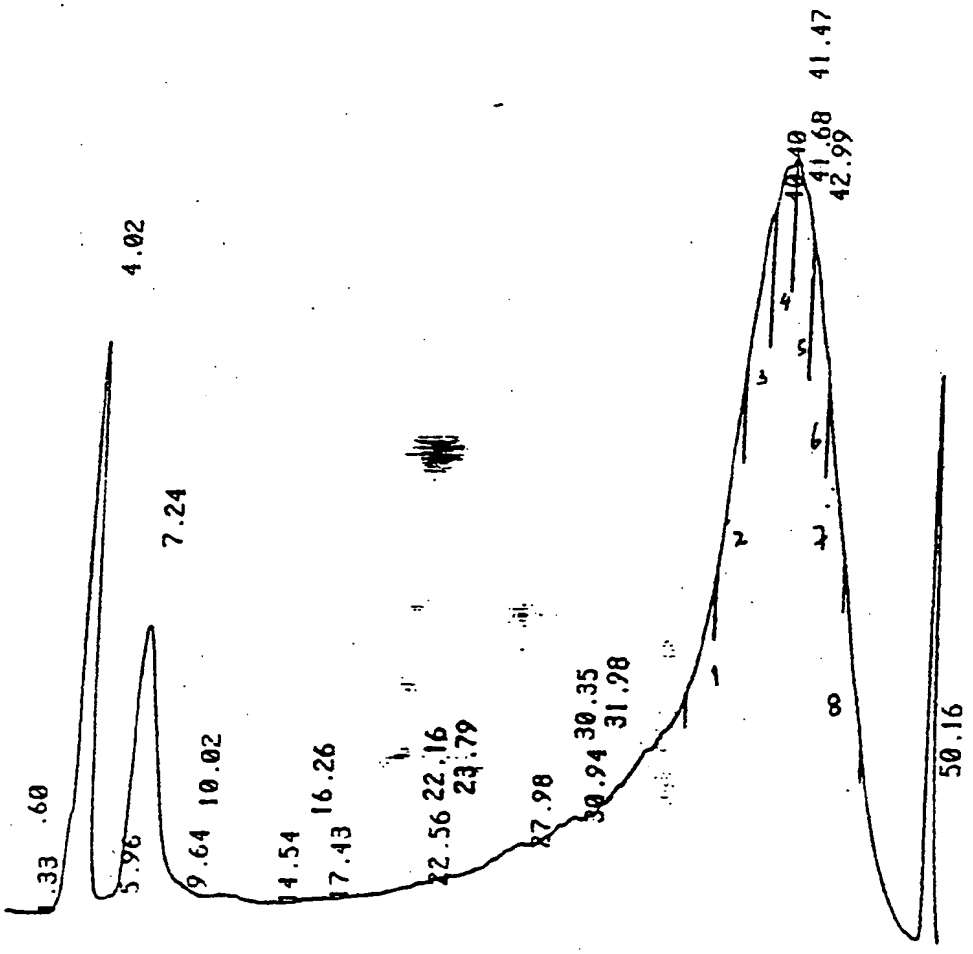

.33
.60
5.96
4.02
9.64
10.02
7.24
4.54
7.43
16.26
22.56
22.16
23.79
27.98
30.94
30.35
31.98
41.47
41.68
42.99
50.16
1
2
3
4
5
6
7
8

FIG. 13

FIG. 14

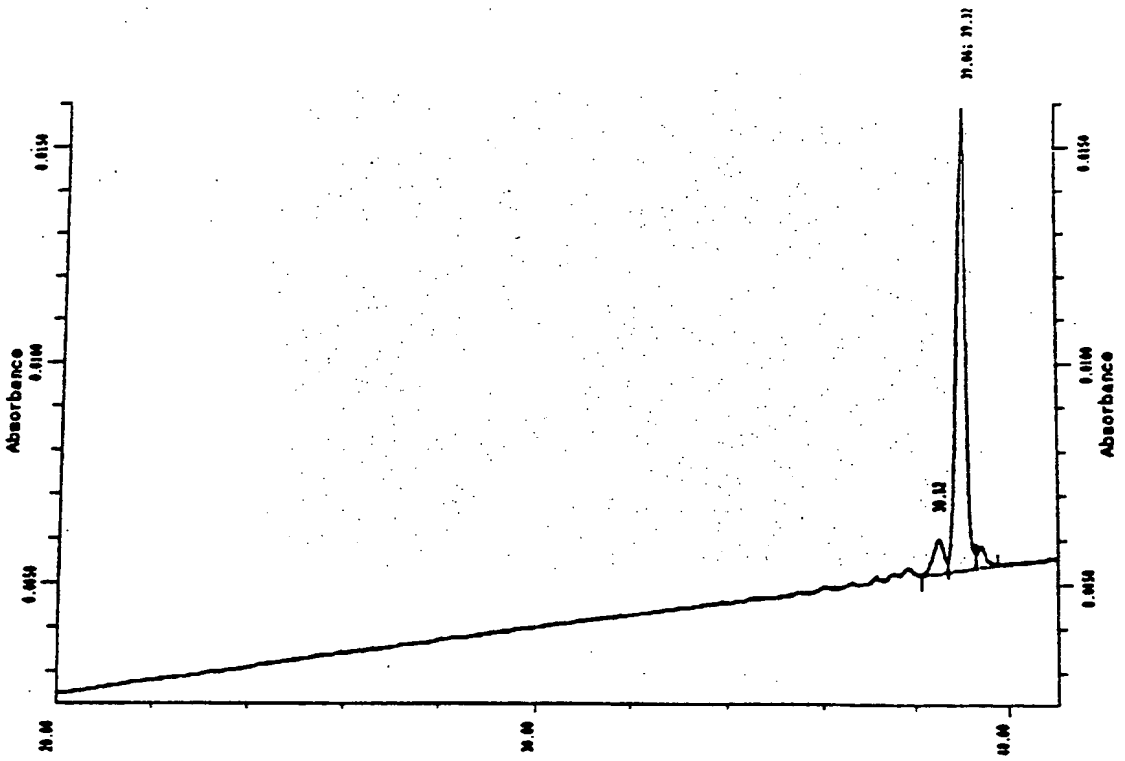


FIG. 15A

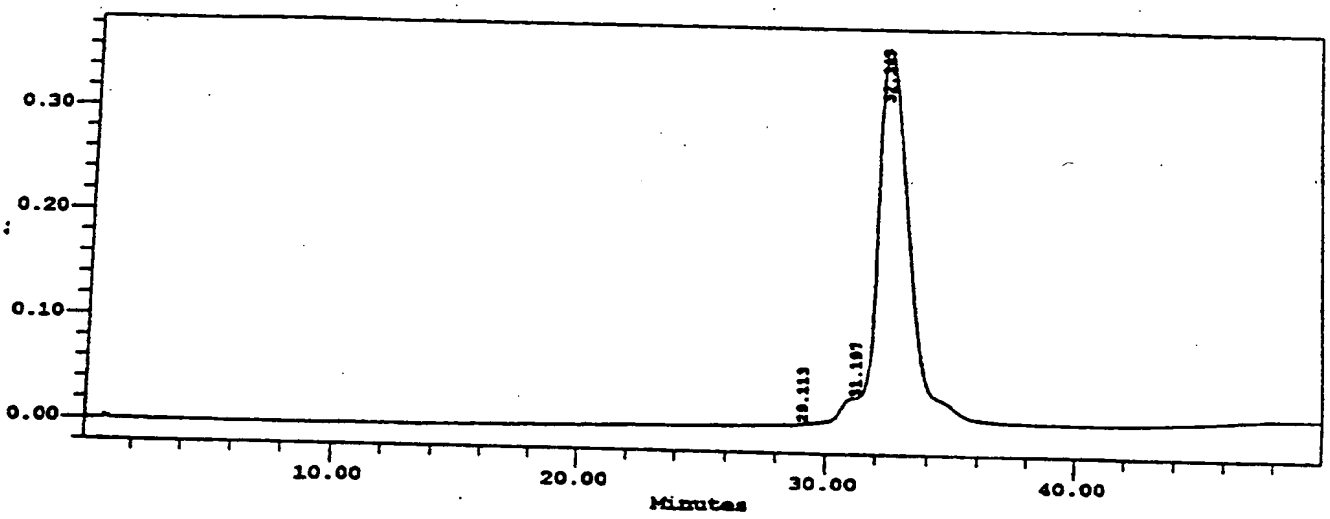


FIG. 15B

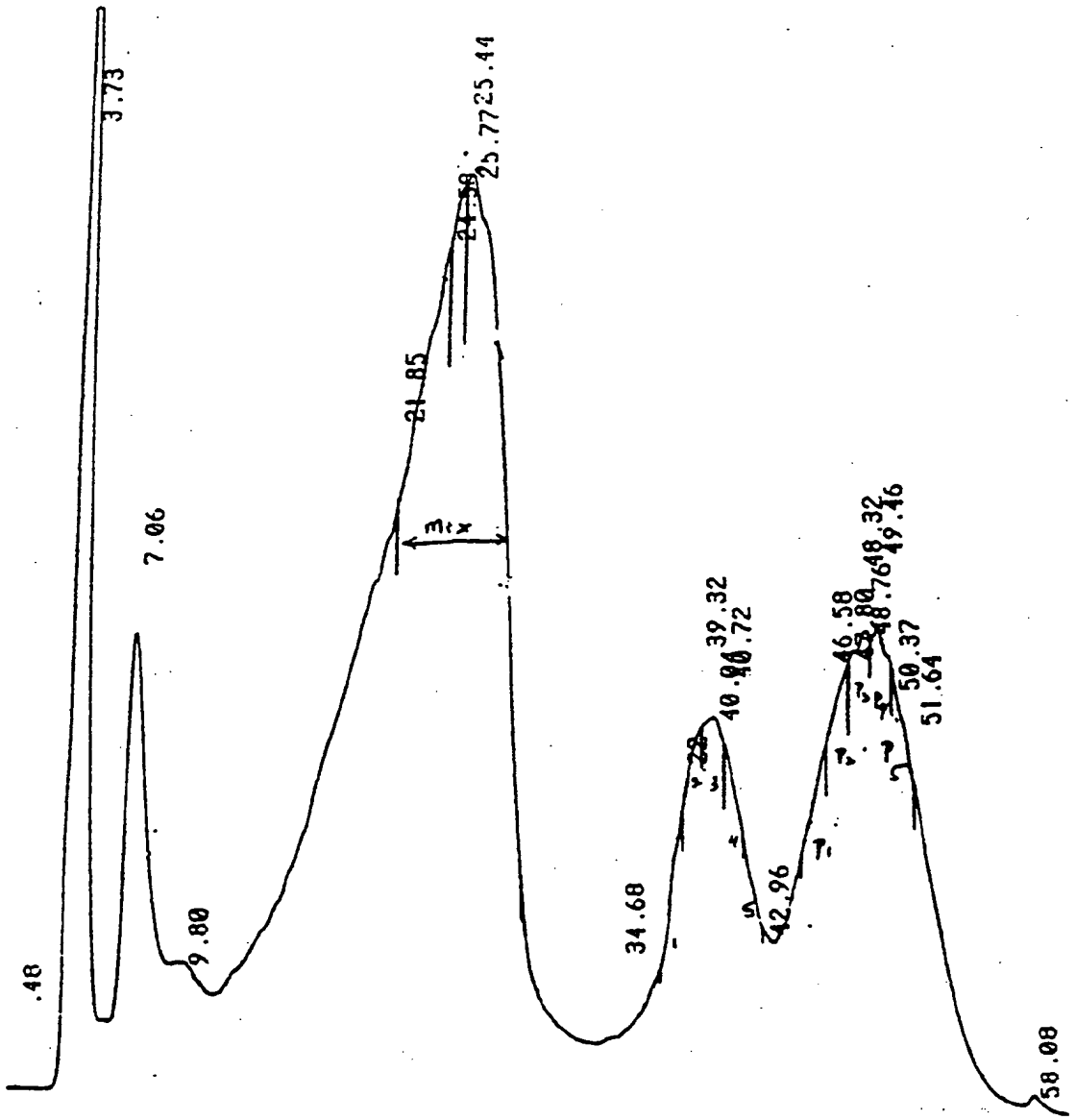


FIG. 16

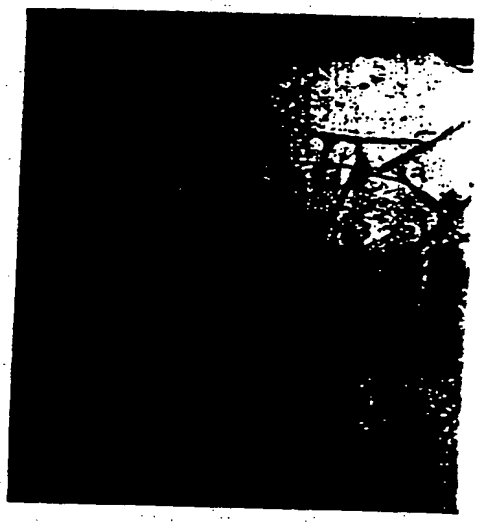


FIG. 17